CONTROL OF NK CELL FUNCTION AND SURVIVAL BY MODULATION OF SHIP ACTIVITY

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application 60/233,661, filed September 19, 2000, and to a U.S. Provisional Application filed on August 23, 2001, under PEPPER HAMILTON LLP Docket Number 114205.2401, entitled "Control of NK Cell Function and Survival by Modulation of SHIP Activity," the disclosure of which is hereby incorporated in its entirety by reference.

SUPPORT

The development of this invention was supported by NIH/NIDDK grant DK54767. The United States Government may have certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to the hematopoietic-specific SH2-containing Inositol Polyphosphatase (SHIP) and its effect in modulating Natural Killer (NK) cell function and survival. Specifically, genetic and pharmaceutical methods are disclosed for the modulation of SHIP activity in order to influence NK cell function. The invention further relates to methods for decreasing immune rejection of histo-incompatible bone marrow grafts and solid organ allografts or xenografts, and methods for screening substances or genetic constructs for their ability to modulate SHIP activity.

BACKGROUND OF THE INVENTION

Activation of phosphatidylinositol 3'-kinase (PI 3-kinase) by growth factors and oncogenes has been implicated as a critical step in mitogenic signaling, cellular transformation and in the prevention of cell death (apoptosis), as described in Cantley et al, Cell 64:281-302 (1991), Kapeller and Cantley. Bioessays 16:565-76 (1994), and Stephens et al, Biochim BiophysActa 1179:27-75 (1993). PI 3-kinase consists of 85 kDa and 110 kDa subunits which associate with receptor tyrosine kinases, other receptors and intracellular signaling molecules in response to survival signals, treatment with growth factors or in

normal or transformed cells. Blockade of PI 3-kinase function either by mutagenesis or with pharmacological inhibitors prevents mitogenic signaling and can enhance apoptosis by blocking the activation of Akt/Protein Kinase B. Further, two products of PI 3-kinase, PtdIns(3,4,5)P_3 (PIP3) and PtdIns(3,4)P_2, increase in cells treated with mitogenic stimuli, as shown by Hawkins, et al. Nature 358:157-910, (1992) and Klippel et al, Molecular and Cellular Biology 16:41174127 (1996). The products of PI 3-kinase are presumed to act as second messengers, as regulators of protein-protein interactions, or recruit other kinases that phosphorylate downstream effectors of PI3K signaling.

Thus, engagement of receptors on the surface of mammalian cells results in the activation of phosphatidylinositol 3-phospate kinase (PI-3 kinase) and phosphorylation of inositol phospholipids on the cytoplasmic side of the membrane. The generation of phosphatidyl inositol (3,4,5) triphosphate (PIP3) by PI-3 kinase contributes to the activation of signaling pathways that drive cell proliferation and/or prevent apoptosis. Removal of the phosphate group from the D5 position of phosphoinositides by the hematopoietic-specific SH2-containing Inositol Polyphosphatase (SHIP) has been identified as an important negative feedback mechanism influencing cell activation and survival in the mammalian hematolymphoid compartment.

SHIP was originally identified based on its ability to bind Shc, Grb2, the FcγRIIB receptor, and by a gene-trapping approach. Through the use of *in vitro* assays, it was demonstrated that SHIP can remove the 5'-phosphate of PIP3 and inositol 1,3,4,5-tetrakisphosphate (IP4) suggesting that SHIP may counteract the activity of PI-3 kinase or prevent the sustained influx of Ca^{2+} into the cell. The tyrosine phosphorylation and membrane recruitment of SHIP in response to receptor stimulation has been demonstrated in a variety of transformed hematolymphoid cell lines. Following activation of hematopoietic cells, SHIP is recruited to the membrane for better access to key substrates. In addition, mounting genetic evidence indicates that SHIP plays an important role *in vivo* as a negative regulator of cell activation in B lymphoid cells, myeloid cells, and mast cells. For example, one study demonstrated that SHIP-/- mice, although viable and fertile, failed to thrive, displaying only a 40% survival rate by 14 weeks of age. Mortality was associated with extensive consolidation of the lungs resulting from infiltration of myeloid cells. Increased numbers of granulocytes-macrophage progenitors were observed in both the bone marrow and spleen. Helgason, CD et al. (1998) "Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span." Genes Dev. 12(11):1610-20. In

another study, SHIP-/- mast cells were found to be more prone to mast cell degranulation than SHIP-/+ or +/+ cells. Huber, M. et al (1998) "The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation." Proc. Natl Acad Sci USA 95(19):11330-5. In a third study, SHIP-/- mice exhibited chronic hyperplasia of myeloid cells which resulted in splenomegaly, lymphadenopathy, and myeloid infiltration of vital organs. Further, neutrophils and bone marrow-derived mast cells from these mice were less susceptible to programmed cell death induced by various apoptotic stimuli or by growth factor withdrawal. Liu, Q. et al. (1999) "SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation any myeloid cell survival." Genes Dev. 13(7):789-91; Liu, Q. et al. (1998) "The inositol polyphoshate 5-phosphatase SHIP is a crucial negative regulator of B cell antigen receptor signalling." J Exp Med 188(7):1333-42.

Together, these results demonstrate that SHIP is an important regulator of cellular responses in mature cells of certain hematopoietic lineages. The above studies were conducted with knockout mice using the traditional approach of neomycin replacement of exon I of the SHIP gene.

Inositol polyphosphate 5-phosphatases were the subject of U.S. Patent 6,090,621 to Kavanaugh et al. "Signaling inositol polyphosphate 5-phosphatases (SIPS)"; PCT WO9710252A1 to Rohrschneider, L.R. "DNA encoding an SH2-inositol phosphatase, a SHC binding protein"; and PCT WO9712039A2 to Krystal, G. "SH2 containing inositol phosphatase."

None of the aforementioned studies have identified a role for SHIP in NK (natural killer) cell function, nor have these studies identified a role for NK cells in graft-versus-hosts disease (GVHD). It would be advantageous for reasons disclosed and described below, to control the activity of SHIP. Methods for controlling SHIP activity, and the benefits and treatments that the instant invention provides in improving bone marrow and solid organ transplants, potentially abrogating marrow graft and solid organ rejection, together with means for screening for substances that modulate SHIP activity, and more, are contained herein as will become apparent to one of skill in the art upon reading the following disclosure.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 illustrates the production of mice with a SHIP deficiency.

FIGURE 2 illustrates flow cytometric analysis of the NK cell compartment of $SHIP^{+/+}$ and $SHIP^{-/-}$ mice.

FIGURE 3 illustrates Wright-Giemsa stained SHIP NK cells in which $SHIP^{-/-}$ cells exhibit abnormal morphology.

FIGURE 4 illustrates flow cytometric analysis of MHC class I receptors expressed by NK cell populations in $SHIP^{-/-}$ mice.

FIGURE 5 illustrates recruitment of SHIP to NK inhibitory receptors and opposition of Akt activation *in vivo*.

FIGURE 6 illustrates inability of adult $SHIP^{-/-}$ NK cells to kill an allogeneic target cell.

FIGURE 7 illustrates the rejection of "missing self," but not histo-incompatible, bone marrow grafts by $SHIP^{-/-}$ mice. Figure 7A shows the growth of A/Sw(H-2s)/Sn donor marrow in $SHIP^{+/+}$ or $SHIP^{-/-}$ (C57BL6/J) hosts (*p=.0006 for $SHIP^{-/-}$ vs. $SHIP^{+/+}$, and **p=.002 for $SHIP^{-/-}$ vs. positive control); Figure 7B shows the growth of BALB/C ($H\text{-}2^d$) (B) donor marrow in $SHIP^{+/+}$ or $SHIP^{-/-}$ (C57BL6/J) hosts (*p=.0001 for $SHIP^{-/-}$ vs. $SHIP^{+/+}$, and **p=.0633 for $SHIP^{-/-}$ vs. positive control); and Figure 7C is an analysis of "missing self" marrow graft rejection in $SHIP^{-/-}$ hosts, showing the growth of $\beta 2m^{-/-}$ donor bone marrow in $SHIP^{+/+}$ (C57BL6/J) or $SHIP^{-/-}$ (C57BL6/J) hosts (*p=.2894 for $SHIP^{-/-}$ vs. $SHIP^{+/+}$, **p=.0001 for $SHIP^{-/-}$ vs. positive control). The positive control for engraftment in (A-C) was syngeneic transplants of C57BL6/J WBM into C57BL6/J hosts (D-donor, H-host).

FIGURE 8 illustrates the abrogation of GVHD disease in $SHIP^{-/-}$ hosts receiving fully-histoincompatible bone marrow grafts.

FIGURE 9 illustrates that receptor blockade (Ly49C) partially restores rejection of histo-incompatible marrow grafts in $SHIP^{-/-}$ mice.

FIGURE 10 illustrates that SHIP associates with killer inhibitory receptors (KIR) in human NK cells *in vivo*.

SUMMARY OF THE INVENTION

The instant invention teaches inhibition of SHIP function for the suppression of NK cell-mediated activities. Such activities include rejection of histo-incompatible marrow and stem cell grafts (e.g. pluripotent, muscle, neural, liver, and other stem cell types) and rejection of organ transplants.

Thus methods are provided for increasing the efficiency of engraftment of an allogeneic bone marrow transplant or solid organ allograft or xenograft, in the treatment of a patient having a disease, such as cancer, autoimmune disease, HIV/AIDS, or a genetic deficiency requiring such a transplant, in which an efficacious amount of a substance that inhibits SHIP function is administered to the patient, optionally in a pharmaceutically acceptable carrier. The invention also provides a method for reducing graft-versus-host-disease following histo-incompatible marrow grafts.

In another embodiment, the invention provides a method for decreasing rejection of a MHC (major histocompatability complex) histo-incompatible marrow graft in a patient, where there is a MHC disparity between donor and patient of 1, 2, 3 or more allele mismatches, or the transplanted marrow is a xenograft (e.g. bone marrow from baboon, chimp, or the like) by administering a substance that inhibits SHIP function and thereby suppresses rejection by impairing NK cell function.

Another embodiment of the invention provides methods for decreasing rejection of a bone marrow allograft, or rejection of a solid organ allograft or xenograft in a patient by administering a substance that inhibits SHIP function.

A further embodiment provides a method for treatment or prevention of graft-versus-host disease in a patient that has, or will, undergo a bone marrow allograft.

A preferred method of the invention further comprises administering to said patient an allogeneic bone marrow transplant.

According to preferred embodiments, the substance suitable for the instant invention can be a nucleic acid, such as a genetic construct or other genetic means directing expression of an antagonist of SHIP function. Nucleic acid molecules suitable for the inventive method include anti-sense polynucleotides, other polynucleotides that bind to SHIP mRNA, recombinant retroviral vector, or a combination thereof. A preferred genetic construct of the invention comprises a gene delivery vehicle, a recombinant retroviral vector, or a combination thereof. In a preferred embodiment, the substance that inhibits SHIP function is a nucleic acid that hybridizes to a SHIP mRNA.

Preferred substances may also include peptidomimetic inhibitors of SHIP function, ribozymes, and an RNA aptamer, or a combination thereof.

Suitable substances for the instant invention may also be a low molecular weight substance having a molecular weight of less than about 10,000 that inhibits SHIP activity.

In yet a further embodiment the invention provides methods for screening substances to identify substances that inhibit SHIP function. A preferred screening method of the invention is through the use of an NK cell line comprising an indicator of SHIP function that is exposed to candidate substances. According to a preferred embodiment, the inventive method for screening a substance suspected of inhibiting SHIP function, comprising: providing an NK cell line that comprises an indicator of SHIP function; contacting said cell line with said substance; and measuring the response of said indicator to said substance, whereby the effectiveness of said substance as an inhibitor of SHIP function is assessed from the response of said indicator.

Preferred indicators include fluorogenic substrates for SHIP, indicators that indicate surface levels of Ly49 receptors, killer inhibitor receptors (KIR), and CD94/NKG2 complexes, Fas, Fas ligand, or phosphatidyl serine in the extracellular leaflet of the plasma membrane.

In one embodiment, the response of said indicator is measured by flow cytometry or by a multi-well fluorescence detector.

According to preferred embodiments, substances that are suitable for screening include a nucleic acid, such as a genetic construct or other genetic means directing expression of an antagonist of SHIP function. Nucleic acid molecules suitable for the inventive method include anti-sense polynucleotides, other polynucleotides that bind to SHIP mRNA, recombinant retroviral vector, or a combination thereof. A preferred genetic construct of the invention comprises a gene delivery vehicle, a recombinant retroviral vector, or a combination thereof. In a preferred embodiment, the substance that inhibits SHIP function is a nucleic acid that hybridizes to a SHIP mRNA. Preferred substances may also include peptidomimetic inhibitors of SHIP function, ribozymes, and an RNA aptamer, or a combination thereof. A suitable substance for the instant invention may also be a low molecular weight substance having a molecular weight of less than about 10,000 that inhibits SHIP activity.

The instant invention further provides a mouse cell comprising a $SHIP^{flox}$ allele of a SHIP gene having a first exon and a promoter, wherein the first exon and the promoter have been deleted in the $SHIP^{flox}$ allele. More preferably, the mouse cell of the invention is

homozygous with regard to the $SHIP^{flox}$ allele. Still more preferably, the mouse cell of the invention is an embryonic stem cell.

The instant invention further provides a transgenic mouse comprising a cell of the invention wherein the cell comprises a $SHIP^{flox}$ allele. In a preferred embodiment, the transgenic mouse of the instant invention is derived from the inventive embryonic stem cell. In a particularly preferred embodiment, the transgenic mouse of the invention has a genotype of $SHIP^{-/-}$. More preferably, the transgenic mouse of the invention does not express SHIP protein.

Further provided are therapeutic compositions comprising a substance that inhibits SHIP function, optionally in a pharmaceutically acceptable carrier.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the novel and unexpected finding that SHIP activity has a critical role in regulating Natural Killer (NK) cell function. The present invention comprises methods for the suppression of allograft and xenograft rejection, prevention of graft-versus-host disease (GVHD) in a patient that has, or will, undergo a bone marrow allograft, and methods for screening substances and genetic therapeutic agents to identify those capable of altering NK cell function.

In view of the following disclosure and Examples, it will be apparent to those of skill in the art that inhibition of SHIP activity is advantageous in the suppression of immune rejection reactions, and in the treatment of GVHD, since the functionality of NK cells is shown herein for the first time to be impaired in an advantageous manner, with respect to immune rejection and GVHD, in the absence of SHIP activity.

Thus in a preferred embodiment, the instant invention provides a method for increasing the efficiency of engraftment of an allogeneic bone marrow transplant, for example in the treatment of cancer, autoimmune disease, HIV/AIDS, or any other genetic impairment that is treated by a marrow transplant. It is known that NK cells have a key role in the rejection of such grafts, and that prolongation of the period before rejection, or elimination of the rejection reaction entirely, are both significantly beneficial for treatment. Thus, in preferred embodiments, a substance that inhibits SHIP function is administered, such as by a genetic construct or as a pharmaceutical, which may be a nucleic acid or other substance that is, or leads to expression of, an antagonist of SHIP function. The genetic construct of the invention is preferably operably linked to a promoter and other signals directing expression of a protein SHIP antagonist, or the antagonist can be an antisense

nucleic acid, or a small molecule enzymatic inhibitor, or a peptidomimetic inhibitor, or a ribozyme.

In still further embodiments, as shown herein, rejection of solid organ allografts or xenografts is decreased by administration of a means for reducing SHIP activity.

The invention further includes embodiments in which the rejection of MHC disparate marrow grafts (i.e. those in which the MHC identity of donor tissue cells may differ from the recipient by 1, 2, 3 or more alleles), is suppressed, thus greatly increasing the probability that suitable donors for a given recipient may be found within a certain population. It should be noted that up to six allele mismatches can be obtained from combinations of alleles selected from the following allele pairs: HLA-A, HLA-B and HLA-C.

In further embodiments, the efficiency of bone marrow transplantation is improved by suppression of graft-versus-host disease in a patient through inhibition of SHIP activity.

In the foregoing methods for enhancing the success of allografts or xenografts, the SHIP antagonist is preferably administered prior to the graft. Administration of, for example, substances that lead to a reduction of SHIP activity can be performed sufficiently long before grafting (for example, for a period of about 1-4 weeks) that an advantageous alteration in the amounts of sub-populations of NK cells is obtained (see, Examples). In this manner, the beneficial effects of SHIP inhibition can be obtained prior to grafting, thereby reducing both the probability of graft rejection and the probability of GVHD, while simultaneously increasing the degree of MHC allelic mismatch that is tolerated.

However, it is not essential to the present invention that means for inhibiting SHIP be administered prior to grafting; beneficial reduction in both the probability of graft rejection and the probability of GVHD, and an increase the degree of MHC allelic mismatch that is tolerated, can still be obtained by administration of a means for inhibiting SHIP activity at, or subsequent to, the time of engraftment.

In yet a further embodiment the invention provides methods for screening substances to identify those substances that inhibit SHIP function. Suitable screening assays for the instant invention may be cellular based. A skilled person will recognize that any cell line that has SHIP activity that could be monitored is suitable for using for the screening assays.

Suitable screening assays may also be performed without the use of a cell culture. For example, a simple chemical reaction is also suitable that assays the impact of a substance being evaluated on the enzyme activity of SHIP. Preferably, an *in vitro* screening method without resorting to a cell culture may use, for example, purified natural or recombinant SHIP enzyme and a suitable substrate that generates a detectable signal when it is cleaved or

otherwise acted upon by SHIP. An example of such a detectable signal is a change in the substrate's fluorescence spectra or intensity. Substances that effect a detectable signal in the presence of SHIP and a substrate are thereby identified, and may be tested for their pharmaceutical effectiveness according to methods well known to those skilled in the art.

In a preferred screening embodiment, methods are provided for screening of substances and genetic constructs that are useful for inhibiting SHIP function. Thus it is envisioned as within the scope of the present invention to use NK cell lines in an assay system that would aid in the screening and identification of pharmaceutical agents or genetic therapies that reduce or eliminate SHIP activity and function.

Such agents or genetic therapies encompass, but are not limited to the following: 1) small molecule inhibitors (preferably having a molecular weight of less than 10,000) of SHIP enzymatic activity (i.e. suicide substrates; competitive or non-competitive inhibitors of SHIP activity; RNA aptamers; or PIP 3, 4, or 5 analogs), 2) anti-sense oligonucleotides, 3) peptidomimetics, 4) ribozymes, 5) means for interfering with transcription and/or translation of SHIP RNA, or 6) genetic therapy comprising transfection with a dominant negative SHIP mutant. These agents and/or genetic therapies can exert their effects by preventing the recruitment of SHIP to complexes with other signal transduction components or to the plasma membrane where SHIP can access its inositol phospholipid substrates. Therefore, such substances are effective by blocking SHIP function in NK cells without necessarily altering enzymatic activity. Because SHIP is an intracellular enzyme, one embodiment of such an assay utilizes a fluorogenic substrate of SHIP that reports SHIP activity. Such fluorogenic SHIP substrates are introduced into NK cell lines, which are either treated with potential inhibitors or left untreated. Such fluorogenic SHIP substrates are, for example, substances that exhibit fluorescence upon cleavage. Methods for preparing such substrates based upon the release from fluorescence quenching that occurs when there is cleavage of a substrate resulting in either (a) the separation of a fluorophore from a fluorescence quenching acceptor, or (b) separation of self-quenching fluorophores, or (c) enhanced fluorescence of a single fluorophore due to changes in its immediate chemical environment subsequent to cleavage, are well known in the art. The relative activity of SHIP is assessed by the fluorescent signal emanating from the cells. NK cells derived from the SHIP-/- mice serve as negative controls for this assay. Further, based on the disclosure herein that loss of SHIP activity results in changes in the surface levels of Ly49 receptors, Fas and Fas ligand (FasL), as well as phosphatidyl serine flipping to the extracellular leaflet of the plasma membrane in NK cells, assays for these markers can serve as additional markers of SHIP inhibition. Therefore, cell-

based assays in NK cells lines permit inhibition of SHIP activity and function to be assessed either directly or indirectly. These assays are monitored by flow cytometry or by multi-well fluorescence detectors to permit whole cell detection of SHIP activity and its reduction by such agents as described above.

Within the present disclosure, the following terms are to be understood as follows.

An "isolated polypeptide" or "isolated polynucleotide" as used herein refers to a polypeptide or polynucleotide, respectively, produced *in vivo* or *in vitro* in an environment manipulated by humans using state of the art techniques of molecular biology, biochemistry and gene therapy. For example, an isolated polypeptide can be produced in a cell free system by automated peptide or polypeptide synthesis, in heterologous host cells transformed with the nucleic acid sequence encoding the polypeptide and regulatory sequences for expression in the host cells, and in an animal into which the coding sequence of the polypeptide has been introduced for expression in the animal. A polypeptide or polynucleotide is "isolated" for purposes herein to the extent that it is not present in its natural state inside a cell as a product of nature. For example, such isolated polypeptides or polynucleotides can be 10% pure, 20% pure, or a higher degree of purity.

The term "inositol polyphosphate 5-phosphatase" as used herein refers to a family of phosphatases each of which removes the 5 phosphate from inositol- and phosphatidylinositol-polyphosphates. The family of proteins is determined by the substrate specificity of these enzymes and by amino acid sequence homology. A description of some of the aspects of the family is provided in Jefferson and Majerus, J Biol Chem 270: 9370-77 (1995).
The term "activated T cell" and "activated B cell" refers to T and B cells that have been stimulated, for example, with cytokines or growth factors, or which have had their antigen receptors cross-linked using antibodies, all of which events stimulate gene expression, cell proliferation or other responses in T and B cells.

The term "tyrosine phosphorylated" as used herein refers to the addition of a phosphate group at a tyrosine residue. Generally, tyrosine phosphorylation of polypeptides is associated with activation or inactivation of signaling pathways. Tyrosine phosphorylation is also associated with activation or inhibition of signaling molecules. Tyrosine phosphorylation of a polypeptide of the invention can occur in response to, for example, B or T cell activation. In some cases, binding to other polypeptides occurs before, after, or during the tyrosine phosphorylation of a polypeptide.

The term "apparent molecular weight" as used herein refers to the molecular weight of the protein or polypeptide as it migrates on a polyacrylamide gel under reducing or non-

reducing conditions. The "apparent" molecular weight may be accounted for by glycosylations or other moieties that alter the molecular weight of the polypeptide alone.

The term "SHIP" as used herein refers to SH2-containing inositol-5-phosphatase. SHIP may have an apparent molecular weight of about 145 kDa and is expressed in at least hemopoietic cells. It contains an amino-terminal src-homology domain (SH2), a central 5'-phosphoinositol phosphatase domain, two phosphotyrosine binding consensus sequences, and a proline-rich region at the carboxyl tail.

The term a "means for inhibiting SHIP function" comprises genetic and non-genetic means for inhibiting SHIP function, and includes substances that inhibit SHIP functions.

Among the genetic construct inhibiting SHIP function are various "gene delivery vehicles" known to those of skill in the art, that facilitate delivery to a cell of, for example, a coding sequence for expression of a polypeptide, such as a SHIP inhibitor, an anti-sense oligonucleotide, an RNA aptamer capable of inhibiting SHIP enzymatic activity, an RNA aptamer capable of inhibiting a ribozyme, or another genetic construct of inhibiting SHIP activity known to those of skill in the art.

Among the non-genetic means inhibiting SHIP function are pharmaceutical agent, pharmaceutically acceptable salts thereof that are preferably administered in a pharmaceutically acceptable carrier.

According to preferred embodiments, substances suitable for the instant invention can be a nucleic acid, such as a genetic construct or other genetic means directing expression of an antagonist of SHIP function. Nucleic acid molecules suitable for the inventive method include anti-sense polynucleotides, other polynucleotides that bind to SHIP mRNA, recombinant retroviral vector, or a combination thereof. A preferred genetic construct of the invention comprises a gene delivery vehicle, a recombinant retroviral vector, or a combination thereof. In a preferred embodiment, the substance that inhibits SHIP function is a nucleic acid that hybridizes to a SHIP mRNA.

Preferred substances may also include peptidomimetic inhibitors of SHIP function, ribozymes, and an RNA aptamer, or a combination thereof.

Suitable substances for the instant invention may also be a low molecular weight substance having a molecular weight of less than about 10,000 that inhibits SHIP activity.

The cell to which said component or substance is delivered can be within a mammal, as in *in vivo* gene therapy, or can be removed from a mammal for transfection, or administration of a pharmaceutical agent, and can be subsequently returned to the mammal, as, for example, in *ex vivo* therapy or *ex vivo* gene therapy. The delivery vehicle can be any

component or vehicle capable of accomplishing the delivery of a gene or substance to a cell, for example, a liposome, a particle, naked DNA, or a vector. A gene delivery vehicle is a recombinant vehicle, such as a recombinant viral vector, a nucleic acid vector (such as plasmid), a naked nucleic acid molecule such as a gene, a nucleic acid molecule complexed to a polycationic molecule capable of neutralizing the negative charge on the nucleic acid molecule and condensing the nucleic acid molecule into a compact molecule, a nucleic acid associated with a liposome (Wang, et al., PNAS 84:7851, 1987), and certain eukaryotic cells such as a producer cell, that are capable of delivering a nucleic acid molecule having one or more desirable properties to host cells in an organism. The desirable properties include the ability to express a desired substance, such as a protein, enzyme, or antibody, and/or the ability to provide a biological activity, which is where the nucleic acid molecule carried by the gene delivery vehicle is itself the active agent without requiring the expression of a desired substance. One example of such biological activity is gene therapy where the delivered nucleic acid molecule incorporates into a specified gene so as to inactivate the gene and "turn off" the product the gene was making, or to alter the translation or stability of the mRNA of the specified gene product. Gene delivery vehicle refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest or of turning off the gene of interest. The gene delivery vehicle will generally include promoter elements and may include a signal that directs polyadenylation. In addition, the gene delivery vehicle can include a sequence which is operably linked to the sequence(s) or gene(s) of interest and, when transcribed, acts as a translation initiation sequence. The gene delivery vehicle may also include a selectable marker such as Neo, SV^2 Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. Gene delivery vehicles as used within the present invention refers to recombinant vehicles, such as viral vectors (Jolly, Cancer Gen. Therapy 1:5164, 1994), nucleic acid vectors, naked DNA, oligonucleotides, cosmids, bacteria, and certain eukaryotic cells (including producer cells; see U.S. Ser. No. 08/240,030 and U.S. Ser. No. 07/800,921), that are capable of eliciting an immune response within an animal. Representative examples of such gene delivery vehicles include poliovirus (Evans et al., Nature 339:385-388, 1989; and Sabin, J. Biol. Standardization 1:115-118, 1973); rhinovirus; pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973); SV40 (Mulligan et al., Nature 277:108-114, 1979); retrovirus (U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242, and WO 91/02805);

influenza virus (Luytjes et al., Cell 59:1107-1113, 1989; McMicheal et al., N. Eng. J. Med. 309:13-17, 1983; and Yap et al., Nature 273:238-239, 1978); adenovirus (Berkner, Biotechniques 6:616-627, 1988; Rosenfeld et al., Science 252:431-434, 1991; WO 93/9191; Kolls et al., PNAS 91:215-219, 1994; Kass-Eisler et al., PNAS 90:11498-11502, 1993; Guzman et al., Circulation 88:2838-2848, 1993; Guzman et al., Cir. Res. 73:1202-1207, 1993; Zabner et al., Cell 75:207-216, 1993; Li et al., Hum. Gene. Ther. 4:403409, 1993; Caillaud et al., Eur. J. Neurosci. 5:1287-1291, 1993; Vincent et al., Nat. Genet 5:130-134, 1993; Jaffe et al., Nat. Genet. 1:372-378, 1992; and Levrero et al., Gene 101:195-202, 1991); parvovirus such as adeno-associated virus (Samulski et al., J. Vir. 63:3822-3828, 1989; Mendelson et al., Virol. 166:154-165, 1988; PA 7/222,684); herpes (Kit, Adv. Exp. Med. Biol. 215:219-236, 1989); SV40; HIV (Poznansky, J. Virol. 65:532-536, 1991); measles (EP 0 440,219); astrovirus (Munroe, S. S. et al., J. Vir. 67:3611-3614, 1993); Semlild Forest Virus, and coronavirus, as well as other viral systems (e.g., EP 0,440,219; WO 92/06693; U.S. Pat. No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic(defective), replication competent viruses (e.g., Overbaugh et al., Science 239:906-910,1988) that nevertheless induce cellular immune responses, including cytotoxic T-cell lymphocytes (CTL).

The term "ex vivo administration" refers to transfecting or administering a substance to a cell, for example a cell from a population of cells that are exhibiting aberrant SHIP activity, after the cell is removed from the mammal. After transfection or administration of the substance, the cell is then replaced in the mammal. *Ex vivo* administration can be accomplished by removing cells from a mammal, optionally selecting cells to transform, rendering the selected cells incapable of replication, transforming or treating the selected cells with a polynucleotide or other means for modulating SHIP activity, and placing the transformed or treated cells back into the mammal.

"Administration" or "administering" as used herein refers to the process of delivering to a mammal a therapeutic agent, or a combination of therapeutic agents. The process of administration can be varied, depending on the therapeutic agent, or agents, and the desired effect. Administration can be accomplished by any means appropriate for the therapeutic agent, for example, by parenteral, mucosal, pulmonary, topical, catheter-based, or oral means of delivery. Parenteral delivery can include, for example, subcutaneous, intravenous, intramuscular, intra-arterial, and injection into the tissue of an organ. Mucosal delivery can include, for example, intranasal delivery. Pulmonary delivery can include inhalation of the agent. Catheter-based delivery can include delivery by iontophoretic catheter-based delivery.

Oral delivery can include delivery of an enteric coated pill, or administration of a liquid by mouth. Administration will generally also include delivery with a pharmaceutically acceptable carrier, such as, for example, a buffer, a polypeptide, a peptide, a polysaccharide conjugate, a liposome and/or a lipid. Gene therapy protocol is considered an administration in which the therapeutic agent is a polynucleotide capable of accomplishing a therapeutic goal when expressed as a transcript or a polypeptide in the mammal.

A "nucleic acid" or a "polynucleotide," as used herein, refers to either RNA or DNA molecule that encodes a specific amino acid sequence or its complementary strand. Nucleic acid molecules may also be non-coding sequences, for example, a ribozyme, an antisense oligonucleotide, or an untranslated portion of a gene. A "coding sequence" as used herein, refers to either RNA or DNA that encodes a specific amino acid sequence, or its complementary strand. A polynucleotide may include, for example, an antisense oligonucleotide, or a ribozyme, and can also include such items as a 3' or 5' untranslated region of a gene, or an intron of a gene, or other region of a gene that does not make up the coding region of the gene. The DNA or RNA may be single stranded or double stranded. Synthetic nucleic acids or synthetic polynucleotides can be chemically synthesized nucleic acid sequences, and can also be modified with chemical moieties to render the molecule resistant to degradation. Synthetic nucleic acids can be ribozymes or antisense molecules, for example. Modifications to synthetic nucleic acid molecules include nucleic acid monomers or derivative or modifications thereof, including chemical moieties, such as, for example, phosphothioate modification. A polynucleotide derivative can include, for example, such polynucleotides as branched DNA (bDNA). A polynucleotide can be a synthetic or recombinant polynucleotide, and can be generated, for example, by polymerase chain reaction (PCR) amplification, or recombinant expression of complementary DNA or RNA, or by chemical synthesis.

The term "an expression control sequence" or a "regulatory sequence" refers to a sequence that is conventionally used to effect expression of a gene that encodes a polypeptide and include one or more components that affect expression, including transcription and translation signals. Such a sequence includes, for example, one or more of the following: a promoter sequence, an enhancer sequence, an upstream activation sequence, a downstream termination sequence, a polyadenylation sequence, an optimal 5' leader sequence to optimize initiation of translation in mammalian cells, a Kozak sequence, which identifies optimal residues around initiator AUG for mammalian cells. The expression control sequence that is appropriate for expression of the present polypeptide differs depending upon the host system

in which the polypeptide is to be expressed. For example, in prokaryotes, such a control sequence can include one or more of a promoter sequence, a Shine-Dalgarno sequence, a ribosomal binding site, and a transcription termination sequence. In eukaryotes, for example, such a sequence can include a promoter sequence, and a transcription termination sequence. If any necessary component of an expression control sequence is lacking in the nucleic acid molecule of the present invention, such a component can be supplied by the expression vector to effect expression. Expression control sequences suitable for use herein may be derived from a prokaryotic source, an eukaryotic source, a virus or viral vector or from a linear or circular plasmid. Further details regarding expression control sequences are provided below. An example of a regulatory sequence is the human immunodeficiency virus ("HIV-1") promoter that is located in the U3 and R region of the HIV-1 long terminal repeat ("LTR"). Alternatively, the regulatory sequence herein can be a synthetic sequence, for example, one made by combining the UAS of one gene with the remainder of a requisite promoter from another gene, such as the GADP/ADH2 hybrid promoter.

"Hybridization" refers to the association of two nucleic acid sequences to one another by specific hydrogen bonding. Typically, one sequence can be fixed to a solid support and the other is free in solution. The two sequences are placed in contact with one another under conditions that favor hydrogen bonding. Factors that affect this binding bonding include: the type and volume of solvent; reaction temperature; time of hybridization; agitation; agents to block the non-specific attachment of the liquid phase sequence to the solid support (Denhardt's reagent or BLOTTO); concentration of the sequences; use of substances to increase the rate of association of sequences (dextran sulfate or polyethylene glycol); and the stringency of the washing conditions following hybridization. See Sambrook et al. MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989), Volume 2, chapter 9, pages 9.47 to 9.57. "Stringency" refers to conditions in a hybridization reaction that favor association of very similar sequences over sequences that differ. For example, the combination of temperature and salt concentration should be chosen that is approximately 12° to 20°C. below the calculated Tm of the hybrid under study.

The term "naked DNA" refers to polynucleotide DNA for administration to a mammal for expression in the mammal or to inhibit SHIP activity. The polynucleotide can be, for example, a coding sequence, and the polynucleotide DNA can be directly or indirectly connected to an expression control sequence that can facilitate the expression of the coding sequence once the DNA is inside a cell. Alternatively, the DNA can direct production of RNA or a polypeptide that inhibits SHIP activity.

"Recombinant retroviral vector" refers to an assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. Preferably, the retroviral vector construct should include a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR. A wide variety of heterologous sequences may be included within the vector construct, including for example, sequences which encode a protein (e.g., cytotoxic protein, disease-associated antigen, immune accessory molecule, or replacement protein), or which are useful in and of themselves (e.g., as ribozymes or antisense sequences). Alternatively, the heterologous sequence may merely be a "stuffer" or "filler" sequence of a size sufficient to allow production of retroviral particles containing the RNA genome. Preferably, the heterologous sequence is at least 1, 2, 3, 4, 5, 6, 7 or 8 Kb in length. The retroviral vector construct may also include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Optionally, the retroviral vector construct may also include selectable markers that confer resistance of recombinant retroviral vector, transduced or transfected, cells to TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more specific restriction sites and a translation termination sequence.

A "therapeutically effective amount" is that amount that will generate the desired therapeutic outcome. For example, if the therapeutic effect desired is reduction or suppression of rejection of a transplant, the therapeutically effective amount is that amount that facilitates reduction or suppression of rejection of a transplant. A therapeutically effective amount can be an amount administered in a dosage protocol that includes days or weeks of administration.

The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such as, for example, a polypeptide, polynucleotide, small molecule (preferably a molecule having a molecular weight of less than about 10,000), peptoid, or peptide, refers to any pharmaceutically acceptable carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity.

"Vector construct" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The vector construct can include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational

modification of messenger, or post-transcriptional modification of protein. In addition, the vector construct must include a sequence which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest and acts as a translation initiation sequence. Optionally, the vector construct may also include a signal which directs polyadenylation, a selectable marker such as Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. In addition, if the vector construct is placed into a retrovirus, the vector construct must include a packaging signal, long terminal repeats (LTRs), and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present).

"Tissue-specific promoter" refers to transcriptional promoter/enhancer or locus defining elements, or other elements which control gene expression as discussed above, which are preferentially active in a limited number of tissue types. Representative examples of such tissue-specific promoters include the PEP-CK promoter, HER2/neu promoter, casein promoter, IgG promoter, Chorionic Embryonic Antigen promoter, elastase promoter, porphobilinogen deaminase promoter, insulin promoter, growth hormone factor promoter, tyrosine hydroxylase promoter, albumin promoter, alphafetoprotein promoter, acetyl-choline receptor promoter, alcohol dehydrogenase promoter, a or P globin promoters, T-cell receptor promoter, or the osteocalcin promoter.

"Mammalian cell" as used herein refers to a subset of eukaryotic cells useful in the invention as host cells, and includes human cells, and animal cells such as those from dogs, cats, cattle, horses, rabbits, mice, goats, pigs, etc. The cells used can be genetically unaltered or can be genetically altered, for example, by transformation with appropriate expression vectors, marker genes, and the like. Mammalian cells suitable for the method of the invention are any mammalian cell capable of expressing the genes of interest, or any mammalian cells that can express a cDNA library, cRNA library, genomic DNA library or any protein or polypeptide useful in the method of the invention. Mammalian cells also include cells from cell lines such as those immortalized cell lines available from the American Type Culture Collection (ATCC). Such cell lines include, for example, rat pheochromocytoma cells (PC12 cells), embryonal carcinoma cells (P19 cells), Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), human embryonic kidney cells, mouse sertoli cells, canine kidney cells, buffalo rat liver cells, human lung cells, human liver cells, mouse mammary tumor cells, as well as others. Also included are hematopoetic

stem cells, neuronal stem cells such as neuronal sphere cells, and pluripotent or embryonic stem cells (ES cells).

The term "antagonist" as used herein refers to a molecule that blocks signaling, such as for example a molecule that can bind a receptor, but which does not cause a signal to be transduced by the receptor to the cell. In the case of inositol polyphosphatase 5'-phosphatases an antagonist might block signaling by binding, for example, at an SH2 domain on the molecule, or by binding, for example, so as to inhibit its phosphatase activity. In general, an antagonist of a polypeptide is an inhibitor of any biological activity of the polypeptide. A given inhibitor or agonist may target and inhibit one biological activity, while not affecting another non-target activity of the molecule.

The instant invention will be better understood by reference to the following non-limiting examples.

EXAMPLE 1 ASSAY METHODS

^{51}Cr release assay

Cell mediated cytolytic activity can be detected with a ^{51}Cr release assay. The percentage of specific lysis of ^{51}Cr -labeled target cells in 200 µl is determined for each lymphocyte population by plotting specific cytotoxicity versus the log 10 of the viable effector number. Spontaneous ^{51}Cr release values vary between 5% and 15% of the total incorporated label.

Specifically, FACS purified 2B4^{+}NK1.1^{+} NK cells from SHIP$^{-/-}$ or SHIP$^{+/+}$ littermates are co-incubated with 5X10^{3} ^{51}Cr-labelled Yac-1 target cells at the indicated effector:target (E:T) ratio in 96-well round-bottomed plates and incubated at 37°C for 5hr. Following incubation half the supernatant in each well is removed to determine radioactivity. The percentage of specific ^{51}Cr release is calculated from the formula 100 x (A-B)/(C-B) where A is ^{51}Cr release in the presence of effector cells and B is the spontaneous release in the absence of effector cells. C is the total ^{51}Cr released from target cells lysed by addition of detergent.

FACS Assays

Following specific fluorescent labeling as indicated, cells are sorted, for example, using a modified Becton Dickenson fluorescence activated cell sorter (FACS II) based on the wavelength of the fluorescent label stain, typically a fluorescein (488 nm) or phycobillidye dye (360 nm, e.g. CPE or APC or equivalent).

EXAMPLE 2 SHIP-/- TRANSGENIC Mice

In order to demonstrate the role that SHIP plays in NK biology, mice are developed in which the first exon and promoter of the SHIP gene are flanked by *loxP* sites, and the first exon is deleted by mating these mice with Cre transgenic mice (Fig. 1A,B). The SHIP genomic locus is isolated from a 129SVJ mouse genomic library (lFIX vector, Stratagene, San Diego, CA), partially subcloned and sequenced to identify the SHIP first exon and genomic regions that flank the SHIP promoter and first exon. A 2.3Kb XbaI-BamHI fragment that is immediately 5' to the SHIP first exon and promoter, a 1.7Kb BamHI fragment containing the SHIP promoter and exon 1 and a 2.8kB BamHI-Sau3A fragment 3' to the first exon are inserted into the pFlox plasmid to yield the SHIP targeting vector. The correct orientation and integrity of these SHIP genomic fragments in pFlox are confirmed by restriction mapping and sequencing. The SHIP targeting vector is then linearized with SspI and electroporated into the TL1 ES cell line and stable integrants selected by culture in the presence of G418. Genomic DNA from G418 resistant ES cell clones is digested with SpeI and XhoI, resolved by electrophoresis, transferred to nitrocellulose and probed with a 0.8kB PstI-KpnI probe that flanks the 3' arm of homology in the SHIP targeting vector. These blots are stripped and reprobed with an HSV-TK cDNA probe to confirm that the ES cell clones with 8.7kB fragment diagnostic of homologous recombination contain only a single integration of the targeting vector. The *neo*/HSV-TK selection cassette, itself flanked by loxP sites (floxed), is removed from homologous recombination ES cell clones by transient expression of the Cre recombinase. ES cell clones harboring this deletion are identified by PCR and Southern blot analysis. ES cell clones with the "floxed" SHIP locus that is prepared for deletion of the SHIP promoter and exon 1 by Cre-mediated deletion are used to generate chimeric mice. Chimeras are intercrossed with C57BL6/J mice and offspring carrying ES cell chromosomes identified by coat color. Offspring that inherited the $SHIP^{flox}$ allele are identified by PCR analysis. $SHIP^{flox}$ mice are mated to a Cre transgenic deleter strain and progeny with the expected deletion of the SHIP promoter and first exon (SHIP null allele) are identified by PCR analysis. The SHIP null allele is then backcrossed to the C57BL6/J background to the F4 generation and F4 $SHIP^{+/-}$ heterozygous mice are intercrossed to yield $SHIP^{+/+}$ and $SHIP^{-/-}$ littermates for test groups. Mice are genotyped by a PCR assay that amplifies genomic DNA prepared from ear punches that are diagnostic for the presence of the null or wild type SHIP alleles or both. The primers that amplify the 5Kb and 0.4kB DNA fragments diagnostic for the wild-type and null alleles, respectively, are

5'-AGTCACGTCCCACCATCCTATG-3' (SEQ ID NO.1) and
3'-CCACAAGTGATGCTAAGAGATGC-5' (SEQ ID NO.2).

The primers that amplify the 0.8kB allele diagnostic for the wild-type allele are

5'-ATG AAG GGT CCC TTG TAG AG-3' (SEQ ID NO.3) and
3'-CTG TGA GCA ACA CTA TTC CC-5' (SEQ ID NO.4).

The cycling conditions for these primers are 94°C for 4 min; followed by 35 cycles at 94°C for 45s, 55°C for 45s, and 72°C for 6min; ending with 10 min at 72°C. Ablation of SHIP expression is confirmed by Western blotting of spleen cells with the anti-SHIP monoclonal (P2C6) that detects all SHIP isoforms.

Mice with germline transmission of the "floxed" SHIP allele ($SHIP^{flox}$ allele) are then mated with transgenic mice that express Cre recombinase in germline gonadal tissues (CMV-Cre) (M. A. Bender *et al.*, *Blood* **92**, 4394-403, 1998). Because of this expression pattern, $CMV\text{-}Cre^{+}SHIP^{flox/+}$ male mice yield progeny with germline transmission of a SHIP null allele due to deletion of the first exon and promoter at the $SHIP^{flox}$ locus.

Fig.1A shows genetic modification of the SHIP locus in mouse ES cells: configuration of **(i)** the wild-type SHIP locus; **(ii)** the targeting vector; and **(iii)** the SHIP locus after homologous recombination by the targeting vector; **(iv)** the deletion of the *neo*/HSV-TK cassette *in vitro* by Cre-mediated recombination results in ES cell clones with a "floxed" SHIP locus ($SHIP^{flox}$) used to generate chimeric mice; and **(v)** the SHIP null allele created by intercrossing of $SHIP^{+/flox}$ mice with Cre deleter mice results in the *in vivo* deletion of the SHIP first exon and promoter in $SHIP^{+/flox}/CMV\text{-}Cre^{+}$ progeny. $SHIP^{+/flox}/CMV\text{-}Cre^{+}$ mice were crossed with C57BL6/J mice and progeny that inherited the SHIP null allele in the absence of the CMV-Cre transgene are identified. Progeny that inherited the SHIP null allele are backcrossed to C57BL6/J out to the F4 generation. Intercrosses of F4 $SHIP^{+/-}$ mice are used to generate all wild-type and null homozygous littermates used in this study. SHIP exon 1 (black rectangle), lengths of diagnostic restriction and PCR fragments and a probe (gray rectangle) used for genotyping are shown. The targeting vector incorporates a *neo*/HSV-TK cassette flanked by loxP sites (black triangles) that allows selection of stable integrants in transfected ES cell clones. B, BamHI; K, KpnI; P, PstI; Sa, SalI; S, SpeI; X, XbaI.

Fig.1B shows a Southern blot of genomic DNA from wild-type ES cells and the homologous recombinant clone G9 digested with SpeI and XhoI. When hybridized with a probe that flanks the 3' arm of homology in the targeting vector (gray rectangle in **A**), DNA from wild type cells shows the expected 23kb band, while DNA from G9 cells shows both the 23b wild type band and the 8.7kb band indicative of homologous integration into the SHIP locus. After hybridization with the 3' flanking probe, the filter is stripped and reprobed with an HSV-TK cDNA probe to confirm that the homologous recombinant clone contains a single integration of the targeting vector.

In Fig.1C, genotyping of intercrosses between $SHIP^{+/-}$ mice is shown. DNA is prepared from ear punches of weanlings and PCR reactions that simultaneously detect both wild-type and null SHIP alleles (upper panel) or only the wild-type allele (lower panel) are performed. This analysis shows that littermates 1 are 3 are null homozygous (-/-), 2 and 6 are heterozygous (+/-) and 4-5 are wild-type homozygous (+/+).

In Fig.1D, Western blot analysis is used to confirm loss of SHIP expression in null homozygous littermates ($^{\#}1, ^{\#}3$). Whole cell lysates from spleen cells are prepared from the litter of mice genotyped in **(C)** and blotted with an anti-SHIP monoclonal antibody (P2C6) that reacts with all SHIP isoforms. Stripping and reprobing of the filter with a monoclonal antibody specific for β-actin shows equal protein loading.

In summary, mice are identified with the SHIP null allele in the germline and backcrossed the SHIP null allele to the C57BL6/J background to the F4 generation. F4 SHIP null heterozygous mice ($SHIP^{+/-}$) are intercrossed to generate $SHIP^{-/-}$ mice and wild-type littermates for the studies described below (Fig. 1C). Most importantly, $SHIP^{-/-}$ mice lack expression of SHIP protein (Fig. 1C,D).

EXAMPLE 3 **Development of an abnormal NK cell population in SHIP-/- mice**

The development of an abnormal NK cell population in SHIP-/- mice is shown, with reference to Fig.2. By 8 weeks of life, SHIP-/- mice show a gross distortion of their NK cell repertoire and a loss of NK cell homeostasis that results in an increased number of NK cells in SHIP-/- mice.

To assess the development of the peripheral NK cell compartment in $SHIP^{-/-}$, mice are analyzed following weaning (3 weeks), at the onset of puberty (5 weeks), and in adult mice (8 weeks or older). To analyze the peripheral NK cell compartment, spleens are collected from mice at various ages, and a single cell suspension is prepared by NH_4Cl lysis of erythrocytes

and stained with the antibodies against the NK-associated markers 2B4 (PE), NK1.1 (FITC) and CD3(APC). For analysis of the NK cell repertoire, cells are stained with 2B4, NK1.1 and anti-Ly49A (A1), -Ly49C/I (5E6), -Ly49D (4E5), -Ly49G2 (4D11) or -CD94 (Ebioscience, San Diego, CA). To distinguish Ly49C staining from Ly49I, cells are stained with NK1.1, Ly49C/I and Ly49I (YLI90). All biotin conjugates are revealed by StrepAvidin-APC. With the exception of the anti-CD94 antibodies and StrepAvidin-APC all FACS reagents are obtained from BD-Pharmingen (San Diego, CA). The statistical significance of FACS analysis is assessed by a two-tailed Students' T-test. Splenocytes are prepared and stained with the NK cell associated markers, 2B4 and NK1.1 (L. L. Lanier, *Annual Review of Immunology* 16, 359-93 (1998); W. M. Yokoyama, *Current Opinion in Immunology* 10, 298-305 (1998)). Analysis of mice at these stages of ontogeny (Fig. 2A) indicate NK cells develop normally in the absence of SHIP expression, but in adult animals an abnormal population of NK cells is present that express approximately 10-fold higher surface levels of the NK receptor, NK1.1 (subsequently referred to as NK1.1^{ht} cells) (Fig. 2A).

Thus, Fig.2. illustrates flow cytometric analysis of the NK cell compartment: (A) Dual color contour plots of splenocytes stained with antibodies against the indicated markers. Results shown are representative of at least three mice from three separate litters. Genotype and age of the mice at the time of sacrifice and analysis are indicated. Fig. 2 Bar graphs indicating the mean percentage of NK cells and the mean absolute number of NK cells in SHIP$^{+/+}$ and SHIP$^{-/-}$ mice at different ages. The values determined for SHIP$^{-/-}$ mice that are significantly different from that of their age-matched SHIP$^{+/+}$ counterparts, are indicated by the following symbols: *, $p<0.05$ and †, $p<0.01$.

The NK1.1^{hi} population lacks CD3 and thus is not an NK-T cell population. Although the NK1.1^{hi} population is most abundant in the spleens of adult mice, it is also detected as a small population as early as 5 weeks of life (Fig. 2A,B). In addition, the relative and absolute number of NK cells with the normal 2B4^{+}NK1.1^{+} staining profile (Fig. 2A) (herein referred to as NK1.1^{+} cells) are also increased in adult SHIP$^{-/-}$ mice (Fig. 2B). Post-weaning SHIP$^{-/-}$ mice (3 weeks) and SHIP$^{-/-}$ mice at the onset of puberty (5 weeks) show no significant increase in the percentage or absolute numbers of NK cells as compared to SHIP$^{+/+}$ littermates (Fig. 2B) indicating that NK cell homeostasis is normal in weanlings and juvenile mice. However, homeostasis is severely disrupted in adults (≥8 weeks) resulting in increased numbers of both NK1.1^{+} cells and the emergence of the abnormal NK1.1^{hi} population that constitutes approximately 30% of the peripheral NK cell compartment in adult SHIP$^{-/-}$ mice (Fig. 2B). Both the NK1.1^{hi} population and increased numbers of NK1.1^{+}

cells are found in all adult SHIP$^{-/-}$ mice examined (8-19 weeks of age). Thus, the loss of homeostasis in adult SHIP$^{-/-}$ mice leads to an approximately three-fold increase in total NK cells in the periphery of SHIP$^{-/-}$mice relative to wild-type littermates (Fig. 2B). Without being limited by theory, the loss of NK cell homeostasis may represent a failure of these cells to die due to unopposed PI3K/Akt signaling.

The alteration in NK cell populations shown herein is accompanied by alteration in the morphology of SHIP-/- NK cells, as shown in Fig.3.

It is thereby shown in this example that the absence of SHIP function greatly influences how NK cells perceive their cellular milieu *in vivo*, and therefore it is shown that SHIP influences normal NK cell function. NK cells actively survey cells for MHC class I and ignore cells that have normal levels of all MHC class I haplotypes. If a departure from normalcy is detected (for example, an MHC class I haplotype normally expressed in the body is missing on a cell) then NK cells kill the aberrant cell: this is known to be how NK cells survey the body for virally infected cells or tumor cells. Such cells as these can lose MHC class I surface expression and thereby avoid T cells.

EXAMPLE 4 **MHC Class I repertoire of SHIP$^{-/-}$ mice**

Receptors that enable self/non-self recognition by lymphocytes play a critical role in their activation and differentiation into effector cells. These receptors also play a critical role in the homeostasis of these lineages through effects on their survival and proliferation in the periphery. Homeostasis in the NK cell compartment of SHIP$^{-/-}$ mice is lost at a time when the NK cell repertoire is normally established, and the repertoire is altered in the NK cell compartment of adult SHIP$^{-/-}$ mice as shown in this Example.

The repertoire of NK receptors for MHC class I molecules in SHIP$^{-/-}$ mice and their wild-type littermates is analyzed at discrete stages of ontogeny. Analysis of the expression of various Ly49 molecules and CD94 in weanlings (3 weeks) (Fig. 4A-D), in juvenile mice (5 weeks) (Fig. 4D) and in adult mice (8 weeks) (Fig. 4A-D) shows that the NK cell repertoire is significantly distorted in older SHIP$^{-/-}$ mice when compared to age-matched SHIP$^{+/+}$ littermates, but not in weanlings. SHIP$^{+/+}$ and SHIP$^{-/-}$ weanlings show no significant difference in the proportion of NK cells that express Ly49A, C/I, D, G2 and CD94 (Fig. 4A,B,D). However, in juvenile mice only two weeks older, the repertoire of the NK compartment of SHIP$^{-/-}$ littermates is distorted toward the expression of Ly49A and C/I (Fig. 4D). This distortion is more pronounced in adult mice (8 weeks) and is found in both the NK1.1^{+} and the NK1.1^{hi} populations (Fig. 4A-D). Both of these NK populations in SHIP$^{-/-}$

mice are 80-90% positive for Ly49A and C/I (Fig. 4A,B), with supernormal levels of these receptors found on the NK1.1^{hi} cells (Fig. 4A). The expression of Ly49D, G2 and CD94 in the NK cell compartment of adult SHIP$^{-/-}$ mice shows the opposite trend with the percentage of NK cells expressing these molecules significantly reduced relative to wild-type littermates (Fig. 4A,B).

Thus, in Fig.4, flow cytometric analysis of MHC class I receptors expressed by NK cell populations in SHIP$^{-/-}$ mice is shown: (A) Histograms indicating expression of various Ly49 receptors or (B) CD94 on peripheral NK cells in SHIP$^{-/-}$ mice and their wild-type littermates. Spleen cells from 3 week old ("weanlings") or 8 week old (adult) SHIP$^{-/-}$ mice and their SHIP$^{+/+}$ littermates are stained with a combination of anti-2B4, anti-NK1.1 and anti-Ly49 or -CD94 antibodies. Fig. 4C histograms showing Ly49I expression on Ly49C^{+} cells in the indicated NK1.1 population of adult SHIP$^{-/-}$ and SHIP$^{+/+}$ littermates. The gate used to calculate the percentage of NK cells that expresses the indicated Ly49 or CD94 molecule is shown by a horizontal black line above each histogram. All histograms are representative of analyses from at least three mice of identical age and genotype. Fig 4D bar graphs indicating the mean percentage of NK cells that express the indicated Ly49 or CD94 molecule as determined in (A). The age and genotype of the mice are indicated. The values determined for SHIP$^{-/-}$ mice that are significantly different from that of their age-matched SHIP$^{+/+}$ counterparts are indicated by the following symbols: *, $p<0.05$ and †, $p<0.01$.

Ly49C/I staining is analyzed on adult NK cells in conjunction with an antibody specific for Ly49I (Fig. 4C), showing that both the NK1.1^{+} and the NK1.1^{hi} populations in adult SHIP$^{-/-}$ mice are predominantly Ly49C^{+}, since only a small proportion of Ly49C/I^{+} cells express Ly49I (Fig. 4C). In contrast, nearly half of the Ly49C/I^{+} NK cells in adult SHIP$^{+/+}$ express Ly49I (Fig. 4C). Thus, the repertoire distortion that occurs in the absence of SHIP signaling leads to an adult NK compartment that is dominated by a subset of cells with the following repertoire: Ly49A^{+}C^{+}D^{-}G2^{-}TCD94^{-}. *In vitro* and *in vivo* studies show that Ly49C and Ly49A can bind H-2^{b} and H-2^{d} class I ligands while Ly49D and Ly49G2 have specificity only for a ligand in the H-2^{d} haplotype. Studies of Ly49A transgenic mice demonstrate that the H-2b haplotype possesses functional inhibitory ligands for Ly49A, since both anti-tumor and anti-viral responses by T cells expressing Ly49A are negatively impacted in the presence of the H-2b haplotype. Taken together, this demonstrates that both Ly49A and Ly49C are capable of binding and transmitting inhibitory signals from ligands present in all major murine MHC class I haplotypes, including H-2b. Thus, the MHC specificity of the NK inhibitory repertoire in adult SHIP$^{-/-}$ mice is both self-specific and promiscuous.

SHIP association *in vivo* with inhibitory Ly49 receptors expressed by NK cells is next shown. NK-enriched C57BL6/J splenocytes are prepared by depletion of B cells and macrophages by adherence to nylon wool followed by T cell depletion using anti-CD3 plus complement. NK cells are then lysed in modified RIPA buffer. Prior to immunoprecipitation the NK cell lysates are pre-cleared by incubation with 0.25μg of an IgG2a antibody (BD Pharmingen) and 80μl of Protein G-Sepharose beads (Amersham Pharmacia). Immune precipitates bound to beads were pelleted at 15,000Xg for 15min at 4°C. The supernatants are immunoprecpiated with Ly49A, Ly49C/I, Ly49G2 and IgG2a by the sequential addition of 1-2μg of the following antibodies to the pre-cleared lysates: anti-Ly49A (A1), anti-Ly49C/I (5E6), Ly49G2 (4D11) and an IgG2a isotype control (BD Pharmingen, San Diego, CA). Immune complexes were brought down by addition of 50μl of Protein G-Sephadex beads. Following each immunoprecipitation, excess antibody is removed by the addition of Protein G-Sephadex beads followed by centrifugation. The immunoprecipitates are resolved on a 4-12% Tris-Bis polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Pharmacia). The filters are then probed with a 1:1000 dilution of anti-SHIP (P2C6) and an anti-mouse IgG secondary antibody (Amersham Pharmacia) at a 1:100,000 dilution. The presence of SHIP is revealed using the SuperSignal West Femto reagent (Pierce). For analysis of Akt activation, lysates of purified NK cells from the spleens of $SHIP^{-/-}$ and $SHIP^{+/+}$ are prepared as above. Equal quantities of protein from cells lysates prepared from $SHIP^{+/+}$ and $SHIP^{-/-}$ NK cells are resolved on a 4-12% Tris-Bis polyacrylamide gel (Invitrogen), transferred to a nitrocellulose membrane (Amersham) and the filters probed with an anti-Akt-P(Threo) antibody (Cell Signalling) at a 1:1000 dilution. The presence of Akt is detected by a donkey anti-rabbit IgG secondary antibody coupled to HRP (Amersham) at a 1:2000 dilution and revealed using ECL substrate (Amersham). The blot is then stripped and reprobed in a similar manner using an anti-β-actin antibody (Cell Signaling) as an internal control for protein loading.

This analysis shows that SHIP associates with Ly49A and Ly49C, but not Ly49G2 (Fig. 5A), under physiological conditions. Thus, in Fig.5, SHIP is shown to be recruited to NK inhibitory receptors and to oppose Akt activation *in vivo*. FIG 5A shows western blot detection of SHIP in Ly49 immunoprecipitates prepared from lysates of NK-enriched C57BL6/J splenocytes. A mock immunoprecipitation of the NK lysates with an IgG2a antibody is analyzed in parallel as a negative control. The results of these immunoprecipitations are representative of two independent analyses of NK-enriched

splenocytes. Fig 5B shows western blot analysis of SHIP in Ly49A and Ly49C immunoprecipitates prepared from lysates of $SHIP^{+/+}$ (+/+) and $SHIP^{-/-}$ (-/-) NK lysates. Immunoprecipitation of SHIP from NK cell lysates serves as positive control in both (**A**) and (**B**). In (**A**) a one-tenth exposure of the SHIP lane enables the 135/145kD SHIP isoforms to be distinguished clearly. Fig 5C shows western blot analysis of Akt activation using an antibody specific for Akt phosphorylated at Threo(408). To control for the amount of cell lysate loaded in each sample, the Akt-P blot is stripped and re-probed with an antibody specific for β-actin. The detection of Akt activation is representative of three separate analyses of NK cell lysates from $SHIP^{-/-}$ and $SHIP^{+/+}$ mice.

As further confirmation that the protein co-precipitating with Ly49A and Ly49C is SHIP, NK lysates from $SHIP^{+/+}$ and $SHIP^{-/-}$ mice are analyzed (Fig. 5B). SHIP is only co-precipitated in the $SHIP^{+/+}$ NK lysates.

Not to be limited by theory, but solely to clarify the possible mechanism of the present invention, SHIP and SHP-1 are both recruited to inhibitory Ly49 receptors, but at different times in the life of an NK cell. SHP-1 may be recruited to these receptors in activated NK cells to prevent inappropriate NK effector functions, while SHIP may influence the survival of specific NK cell subsets *in vivo* by counteracting the PI3K/Akt pathway that promotes their survival. Consistent with this, Akt/Protein Kinase B is activated in $SHIP^{-/-}$ NK cells *in vivo* based on its phosphorylation at Threonine 408, while Akt in $SHIP^{+/+}$ NK cells shows only basal level activation (Fig. 5C). Thus, SHIP can oppose activation of Akt in NK cells *in vivo*. In performing this function, SHIP likely prevents the survival and inappropriate expansion of specific NK subsets that express inhibitory receptors capable of recruiting SHIP to the membrane. This mechanism is consistent with the repertoire disruption seen in $SHIP^{-/-}$ mice where 90-95% on adult NK cells co-express Ly49A and Ly49C.

Significantly, we have shown that there exists a similar association between SHIP and human Killer Inhibitory Receptors (KIR), the human equivalent of the mouse MHC Class I Ly49 inhibitory receptors Ly49. Specifically, as shown in Figure 10, human NK cells were enriched from peripheral blood mononuclear cells by magnetic depletion of B cells, T cells, monocytes, granulocytes and red blood cells with a cocktail of anti-CD19, -CD3, -CD4, -CD66b, and glycphorin A (StemSep, Vancouver), and the NK enriched fraction was lysed in RIPA buffer. Anti-bodies specific for the indicated KIR molecules and ProteinG+A-sepharose were used to immunoprecipitate the KIR molecules from human NK cell lysates.

The immunoprecipitates were then resolved by SDS-Page and transferred to a blotting membrane. A Western blot of the immunoprecipitates indicates that SHIP is associated with some KIR (KIR-NKAT2, NKB1) in this individual. Mock immunoprecipitations with antibody isotype matched controls for the indicated KIR antibodies failed to immunoprecipitate SHIP (data not shown). Again, not to be limited by theory, the data in Figure 10 show that SHIP is likely to influence signals that affect NK subset survival or proliferation and function via KIRs, probably because the cytoplasmic tails of both Ly49 and KIR have conserved ITIM motifs to which SHIP binds to allow its recruitment to the membrane and access to its substrate, PIP3.

EXAMPLE 5 **NK cell function in SHIP$^{-/-}$ mice, and effect upon graft rejection**

The severe distortion of the NK cell repertoire towards receptors with promiscuous specificity for ligands from many different MHC haplotypes increases the inhibitory signals received by these cells and may hamper their function. The ability of SHIP$^{-/-}$ and SHIP$^{+/+}$ NK cells from juvenile and adult mice to carry out cytolysis of an NK-sensitive allogeneic target cell (YAC-1) is assayed in this Example.

YAC-1 cells are derived from A/Sn mice that have an H-2a haplotype. The results in Figure 6 shows that there is no significant difference in the ability of wild-type and mutant NK cells from juvenile mice (5 weeks) to lyse target cells. However, purified SHIP$^{-/-}$ NK cells from adult mice (8 weeks) show severely reduced lysis of YAC-1 targets (Fig. 6). Splenic 2B4^{+}NK1.1^{+} NK cells were purified by FACS and analyzed for their ability to lyse an NK-sensitive target cell (YAC-1) in a standard ^{51}Cr release assay at the indicated effector:target ratios (E:T). The percent specific lysis of target cells by NK cells from SHIP$^{-/-}$ and SHIP$^{+/+}$ littermates of the indicated ages are shown in FIG 6. The results are representative of three independent experiments using SHIP$^{+/+}$ and SHIP$^{-/-}$ littermates from three different litters. Adult SHIP$^{-/-}$ NK cells enriched following nylon wool depletion of adult splenocytes also fail to kill target cells.

SHIP$^{-/-}$ NK cells, however, showed reduced capacity to kill normal cells of the H-2s and H-2d MHC backgrounds. Whole bone marrow (WBM) cells are obtained from tibias and femurs of A/SW-(H-2s)/Sn (H-2s), BALB/C (H-2d) or β2m$^{-/-}$ donor mice and washed once in PBS. WBM cells (5X10^{6}) are injected intravenously into lethally irradiated hosts (950 Rad). After 5 days, 3μCi of 5'-[^{125}I]iodo-2'-deoxyuridine (^{125}I-dUrd) is injected intravenously. The next day mice are sacrificed, their spleens removed and the incorporated radioactivity

measured. The statistical significance of differences in the means between experimental groups is assessed by a two-tailed Students' T-test. For analysis of survival and GVHD (Graft Versus Host Disease) following allogeneic marrow transplantation, $5X10^6$ WBM cells are transplanted into mice that received 950Rads as a single dose. The mice are kept on acidified water for the first 4 weeks post-transplant. Mice are weighed two times per week for the first 6 weeks and then weekly. Mice are observed daily for evidence of severe GVHD including hunched posture, alopecia, inflammation or bleeding of mucous membranes during the first four weeks post-transplant and then twice weekly.

As shown in Fig. 7A, $SHIP^{-/-}$ mice are permissive for the growth of A/Sw(H-2s)/Sn marrow grafts while their $SHIP^{+/+}$ littermates reject these grafts. The inability of $SHIP^{-/-}$ NK cells to reject A/Sw(H-2s)/Sn marrow grafts is primarily due to the co-expression of Ly49A and Ly49C by an overwhelming proportion of the adult $SHIP^{-/-}$ NK cell compartment. Consistent with this proposed mechanism, the H-2s haplotype is also capable of binding and/or transmitting inhibitory signals via either Ly49A or Ly49C. Ly49D, an activating receptor that is down regulated in $SHIP^{-/-}$ mice, does not have a ligand in the H-2s haplotype and thus its under-representation in $SHIP^{-/-}$ mice is not responsible for acceptance of H-2s marrow grafts.

NK killing of other histo-incompatible targets is also compromised in $SHIP^{-/-}$ mice, including killing of cells bearing other MHC haplotypes that bear MHC ligands bound by Ly49A and Ly49C. $SHIP^{-/-}$ mice cannot reject a fully allogeneic bone marrow graft from BALB/C mice whose H-2d haplotype forms strong interactions with both Ly49A and Ly49C. BALB/C marrow is not rejected by $SHIP^{-/-}$ mice, but their wild type littermates reject these fully histo-incompatible marrow grafts (Fig. 7B). Thus, $SHIP^{-/-}$ mice fail to reject allogeneic marrow grafts from either H-2d or H-2s donors, consistent with the observation that Ly49A transgenic mice on an H-2b background also fail to reject BALB/C marrow grafts.

Because Ly49A and Ly49C are highly promiscuous receptors capable of interactions with all major murine MHC haplotypes, $SHIP^{-/-}$ mice may be universal recipients for histo-incompatible marrow grafts of any MHC haplotype.

This is supported by the data in Figure 9, which shows that *in vivo* blocking of Ly49C partially restores the ability of $SHIP^{-/-}$ mice to reject BALB/C(H-2D) marrow grafts. Specifically, anti-Ly49C F(ab')2 fragments were injected into SHIP-/- (Null) and SHIP+/+ (WT) recipients 18hr prior to lethal irradiation and these mice were transplanted with 2.5X10 6 whole bone marrow (WBM) cells. Five days later the mice were injected with 3μCi of

125I-UdR. The next day their spleens were removed and counted in a gamma counter to determine the degree of marrow growth. Mice treated with the F(ab')2 fragment show significantly reduced growth of BALB/C marrow (*Null Serum vs Null Fab, p= 0.0476) relative to SHIP-/- mice treated with normal mouse serum (Serum), indicating a partial restoration of marrow rejection due to blocking of the Ly49C receptors over-represented in the SHIP-/- NK compartment. The effect is only partial, since SHIP-/- NK cells also over-express Ly49A and thus this inhibitory receptor may still render some SHIP-/- NK cells unresponsive to the allogeneic marrow cells. Positive control is C57BL/6 marrow transplanted into lethally irradiated C57BL/6 hosts. P-values determined by a Mann-Whitney U-test.

An alternative explanation for the lack of an NK cell response against allogeneic targets is that the NK compartment in adult $SHIP^{-/-}$ mice is impaired. To exclude this possibility, the ability of $SHIP^{-/-}$ mice to reject a "missing self" marrow graft (MHC class I negative marrow from $\beta 2m^{-/-}$ mice) is shown. Fig. 7C shows analysis of 10 separate adult $SHIP^{-/-}$ mice which reject $\beta 2m^{-/-}$ marrow grafts as do their 10 $SHIP^{+/+}$ littermates.

Thus, despite their inability to reject fully histo-incompatible marrow grafts, adult $SHIP^{-/-}$ NK cells still retain cytolytic activity against "missing self" targets *in vivo*. This is explained by the failure of $\beta 2m^{-/-}$ target cells to engage Ly49A and C on $SHIP^{-/-}$ NK cells leading to killing, whereas these receptors are engaged by MHC ligands on allogeneic targets to prevent killing by SHIP-/- NK cells.

EXAMPLE 6 **Inhibition of SHIP prevents rejection of fully histo-incompatible marrow grafts and prevents graft-versus-host disease**

The above Examples in an acute transplant setting demonstrate that $SHIP^{-/-}$ mice fail to reject a fully histo-incompatible marrow graft, but do not address whether engraftment of the donor marrow will result in severe graft-vs.-host disease (GVHD) and death. To address this question, a cohort of $SHIP^{-/-}$ mice and their $SHIP^{+/+}$ littermates were transplanted with whole bone marrow from BALB/C mice following lethal irradiation (Fig.8). GVHD disease is abrogated in $SHIP^{-/-}$ hosts receiving fully-histoincompatible bone marrow grafts based on their enhanced survival and the absence of severe GVHD symptom. (Fig. 8A) Survival of $SHIP^{-/-}$ (n=14) and $SHIP^{+/+}$ (n=14) recipients on the C57BL6/J background that are transplanted with $5X10^6$ WBM cells from BALB/C mice. Mice receive 950Rads prior to BM transplant. (Fig.8B) FACS analysis of donor vs. host re-population for B cells ($B220^+$),

myelo-granulocytic cells (Mac-1$^+$/Gr-1$^+$) or T cells (CD3$^+$) in peripheral blood of a representative SHIP$^{-/-}$ BMT survivor from (A). The rectangular gate used to assess the degree of donor (D) contribution to each lineage post-BMT is shown within the dual contour plots. (C) Donor repopulation of B cells, T cells and myelo-granulocytic cells in the 12 surviving SHIP$^{-/-}$ and 5 surviving SHIP$^{+/+}$ recipients in (A) as determined by multi-parameter FACS analysis of their peripheral blood (B).

Eighty-six percent (86%) of the SHIP$^{-/-}$ mice survive lethal irradiation without developing GVHD out to 10 weeks post-transplant while only 36% survived in the SHIP$^{+/+}$ cohort. Analysis of the survival differences between the two cohorts using the Kaplan-Meier log-rank test confirms that survival of SHIP$^{-/-}$ mice is dramatically enhanced relative to their SHIP$^{+/+}$ littermates (p=0.007) (Fig. 8A). Nine of fourteen SHIP$^{+/+}$ mice died during the 10 week post-transplant period and prior to death exhibit one or more signs of severe GVHD, including hunched posture, alopecia, weight loss and inflamed mucosal tissues. The 12 of 14 surviving SHIP$^{-/-}$ mice show no evidence of severe GVHD up to 10 weeks post-transplant. To show that the transplanted mice are repopulated by BALB/C marrow, donor reconstitution by FACS at 7 weeks post-transplant is assessed (Fig. 8B) and it is found that 11 of 12 surviving SHIP$^{-/-}$ mice have full donor reconstitution of B-lymphoid and myelo-granulocytic cells (Fig. 8C) consistent with engraftment by stem/progenitor cells from the BALB/C marrow graft. The remaining SHIP$^{-/-}$ survivor is reconstituted by both host and donor stem/progenitor cells. Nearly all SHIP$^{-/-}$ hosts showed significant donor T cell reconstitution (Fig. 8C). The enhanced survival of SHIP$^{-/-}$ hosts demonstrates that SHIP not only plays a role in acute rejection of histo-incompatible marrow grafts by NK cells, but that SHIP also influences host factors that contribute to GVHD.

Because SHIP$^{-/-}$ NK cells fail to respond to histo-incompatible marrow grafts, (Fig. 7) and fail to develop GVHD, host NK cells are implicated in the initiation of GVHD. SHIP$^{-/-}$ mice reject "missing self" bone marrow grafts, but not histo-incompatible bone marrow grafts. (Fig. 7 A, B).

NK cells responding to allogeneic targets produce inflammatory cytokines (γ-IFN, TNF-α) that contribute to GVHD. Not to be limited by theory, SHIP$^{-/-}$ NK cells fail to produce inflammatory cytokines in response to these grafts, thereby reducing the likelihood of a significant GVH reaction.

The expansion of an NK cell subset that expresses multiple Ly49 receptors specific for self MHC ligands in adult SHIP$^{-/-}$ mice means that SHIP signaling acts to prevent the

survival or proliferation of such cells *in vivo*. Although Ly49 inhibitory receptors can block NK cell effector function, the interaction of these receptors with self MHC ligands also elicits signals that promote the survival or proliferation of these cells *in vivo*. Thus, SHIP counteracts these pathways and prevents the expansion of NK cells that express multiple self-specific MHC class I inhibitory receptors. SHIP performs this function in NK cells by opposing the PI3K/Akt pathway that influences survival and proliferation of both lymphoid and myeloid cells. Thus, it is shown herein that pharmaceutical inhibition of SHIP signaling limits the NK cell repertoire to subsets that co-express receptors specific for self MHC ligands. Therefore, it is further shown that modulating the NK repertoire in this manner compromises the host NK cell response to histo-incompatible marrow grafts that share some subset of host MHC class I ligands and thus facilitates engraftment in the absence of GVHD. Thus, inhibition of SHIP signaling is a means to increase the efficacy and utility of allogeneic bone marrow transplantation.

Various publications, U.S. and foreign patent documents have been referred to herein, and each is hereby incorporated in its respective entirety by reference.

While the invention has been described in terms of various preferred embodiments, those skilled in the art will recognize that various modifications, substitutions, omissions and changes can be made without departing from the spirit of the present invention. Accordingly, it is intended that the scope of the present invention be limited solely by the scope of the following claims.